DIGE approved

Proteomics in ADME/Tox studies: Automated spot handling of peptides prior to MALDI and electrospray MS identification of proteins altered by drug treatment

key words:
Ettan Spot Handling Workstation • protein identification • Ettan DIGE system • sample preparation • MALDI-ToF MS • LC-MS/MS

This application describes the use of the Ettan™ proteomics platform to produce biologically significant proteomics data. Samples from livers of mice either treated with a candidate drug or untreated were quantified, labeled, and subjected to 2-D gel electrophoresis. Following 2-D gel scanning and imaging, pick lists that included protein spots differentially regulated following treatment with the candidate drug were created for unattended, reproducible spot handling on Ettan Spot Handling Workstation. Subsequent mass spectrometry using MALDI-ToF, chemically assisted fragmentation-MALDI, and LC-MS/MS resulted in confident identification of 100% of the proteins showing at least 50% changed level after treatment with the drug candidate.

Introduction

It is estimated that about 50% of drugs in development fail during clinical trials because of deficiencies in their ADME/Tox (absorption, distribution, metabolism, elimination, and toxicity) properties (1). The cost of these failures so late in the drug development process is naturally very high. In addition, it has been suggested that over 5% of hospitalized patients still suffer serious adverse reactions to drugs that have successfully completed their development and come onto the market (1). Improved means of gathering ADME/Tox information earlier in drug development should thus benefit pharmaceutical manufacturers and, of course, patients.

We have investigated whether a proteomics approach would identify differential levels of mouse liver proteins after treatment of mice with a candidate drug. Such information could help elucidate which proteins are involved in metabolism and toxicity and thus increase the value of ADME/Tox studies in drug discovery.

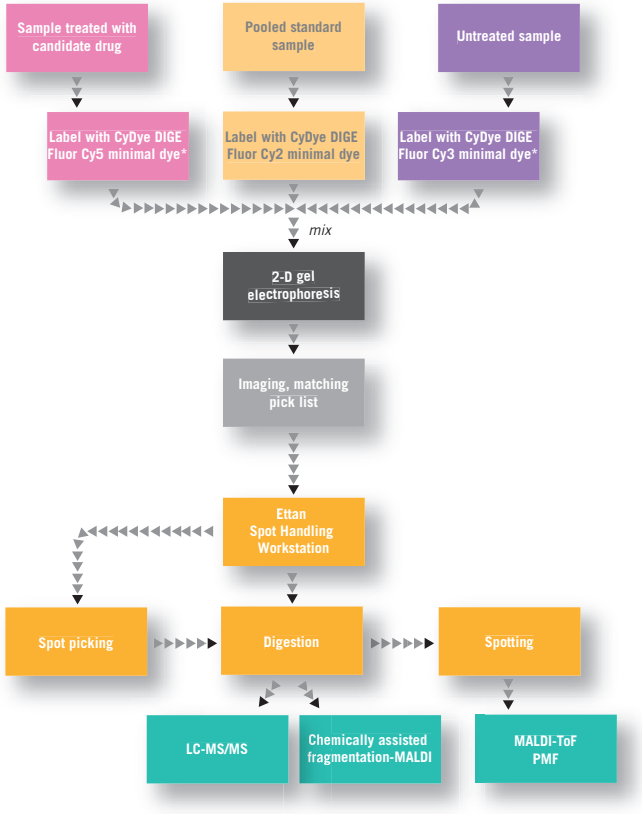

Fig 1. The experimental workflow used for the successful (100%) identification of proteins selected by the criterion of displaying at least a 50% change in abundance after treatment with the candidate drug. * In the experimental workflow, a second gel was run where the labelling was reversed i.e. treated sample was labeled with Cy3 and the untreated sample labeled with Cy5 CyDye DIGE Fluor minimal dye respectively.

Amersham Biosciences

The Ettan design platform offers a complete solution of reagents, kits, and systems for proteomics-based research at a stage before biological validation is feasible. Central to the whole proteomics workflow is the Ettan Spot Handling Workstation, a highly flexible and robust system that allows fully-automated, unattended low- and/or high-throughput handling of protein spots separated by 2-D gel electrophoresis.

A key feature of the workstation is that it avoids the manual errors that normally have a negative impact on obtaining reproducible results. Avoiding such errors is of critical importance since downstream biological validation of the data obtained is very labor intensive. Every effort made to gather reproducible and accurate data at the proteomics experimental stage will pay dividends later. In other words, it is essential that the "right" spots are chosen and picked for downstream validation studies.

Figure 1 illustrates the full experimental workflow and key techniques used in the investigation, which are described in more detail in the Methods section.

Products used

Amersham Biosciences products used:

Ettan sample preparation kits and reagents	
2-D Clean-Up Kit	80-6484-51
2-D Quant Kit	80-6483-56
DeStreak Reagent	17-6003-19
2-D electrophoresis systems and consumables	
CyDye DIGE Fluor Cy2 minimal dye, 25 nmol	RPK0272
CyDye DIGE Fluor Cy3 minimal dye, 25 nmol	RPK0273
CyDye DIGE Fluor Cy5 minimal dye, 25 nmol	RPK0275
Immobiline DryStrip, pH 3–10 NL 24 cm	17-6003-77
Ettan IPGphor II IEF System	80-6505-03
Ettan DALT*twelve* Large Vertical Electrophoresis System	80-6466-27
Typhoon 9410 Variable Mode Imager	60-0055-81
DeCyder Differential Analysis Software	18-1163-05
Spot handling and mass spectrometry	
Ettan Chemicals Trypsin, Sequencing Grade	17-6002-75
Ettan Chemicals Trifluoroacetic Acid,	17-6002-76
Ettan Spot Handling Workstation	18-1164-05
Ettan MALDI-ToF Pro	18-1156-53
Ettan CAF MALDI Sequencing Kit	17-6002-97

Other materials required

SYPRO Ruby stain (Molecular Probes)
Alpha-cyano-4-hydroxycinnamic acid (LaserBio Labs)
Biobasic C18 column (Thermo Hypersil)
Finnigan ProteomeX Workstation (Thermo Electron Corporation)
Finnigan LTQ Linear Ion Trap Mass Spectrometer (Thermo Electron Corporation)
Finnigan LCQ Deca XP Plus Ion Trap Mass Spectrometer (Thermo Electron Corporation)

Methods

Treatment of mice with candidate drug

The selected candidate drug was administered orally to inbred C57BL/6 mice over five days. Livers from treated mice and untreated littermates were removed, dissected, and snap frozen in liquid nitrogen.

Sample preparation and quantitation

Sample (0.5 g) of each liver (three treated livers and one pooled control each of three untreated livers) were rinsed in PBS and homogenized in 10 volumes (5 ml) lysis buffer (10 mM Tris-HCl pH 8.3, 7 M urea, 2 M thiourea, 5 mM magnesium acetate, 4% CHAPS) according to reference 2.

To remove interfering nonprotein material, 2-D Clean-Up Kit was used on a 10 × 100 µl homogenate according to the kit instructions. 2-D Quant Kit was used to quantitate the prepared samples, also according to the kit instructions.

Sample labeling

Samples were thawed and their pH determined by pipetting 1 µl of each onto pH paper. The pH was adjusted to between pH 8.0 and pH 8.5 by adding 2 µl of 50 mM NaOH to each 100 µl aliquot of homogenate. One-hundred microgram of each sample was labeled according to reference 3. Individual samples (treated and untreated livers) to be analyzed were labeled with CyDye™ DIGE Fluor Cy™5 minimal dye and CyDye DIGE Fluor Cy3 minimal dye respectively. In addition, a pooled standard containing all samples included in the experiment was prepared and labeled with CyDye DIGE Fluor Cy2 minimal dye.

The differently labeled individual samples and the pooled standard were mixed prior to 2-D electrophoresis.

2-D gel electrophoresis

First dimension: Cup loading was selected for analytical electrophoresis. One-hundred and fifty microgram of the mixed sample (50 µg of each Cy2, Cy3, and Cy5-labeled sample on each gel) was applied to each 24-cm Immobiline DryStrip, pH 3–10 NL. For preparative electrophoresis, 1 mg samples were loaded onto 24-cm Immobiline™

DryStrip gels, pH 3–10 NL by in-gel rehydration in DeStreak™ Rehydration solution + 2% IPG buffer, pH 3–10. The rehydration step was performed according to the user manual.

Second dimension: Electrophoresis (2 W/gel) was performed overnight using lab-cast gels (4) on Ettan DALT*twelve* electrophoresis system according to the protocols in reference 5. The preparative gels were poststained by SYPRO™ Ruby according to the manufacturer's instructions.

Scanning

Each gel was scanned at 100 µm resolution on Typhoon™ 9410 Variable Mode Imager using 520BP40 (Cy2), 580BP30 (Cy3), and 670BP30 (Cy5) emission filters.

Image analysis

Images were analyzed using DeCyder Differential Analysis Software, v5.0 in both the DIA and the BVA modules. For statistical analysis, two-way ANOVA was used with the Student's t-test value set to 0.01 ± 1.5 times intensity. Individual images were created for the different Cy2, Cy3, and Cy5 labeled gels and matched using DeCyder software. Two preparative gels were added to the experimental study and parallel pick lists that included all spots of interest (i.e. those differentially regulated following treatment with the candidate drug) were created for subsequent spot handling prior to mass spectrometry.

Automated spot handling

Selected proteins were subjected to fully automated spot handling in Ettan Spot Handling Workstation. A method that included spot picking, digestion, and spotting on Ettan MALDI target slides was selected in the software, and the whole procedure was run automatically overnight without any manual intervention either within or between steps.

In this automated procedure, gel plugs were cut by a 2-mm picking head and washed twice in 50% methanol and 50 mM ammonium bicarbonate and once in 75% acetonitrile before drying. For digestion, 10 µl trypsin solution (0.2 µg Trypsin, Sequencing Grade, [Ettan Chemicals]) was added before incubation at 37 °C for 2 h. Extraction was performed in two steps by addition of 50% acetonitrile and 0.1% Trifluoroacetic Acid (Ettan Chemicals). The pooled extract was finally dried prior the two-step spotting procedure, where the matrix solution of 5 mg/ml solution of recrystallized α-cyano-4-hydroxy-cinnamic acid (LaserBio Labs) in extraction liquid was deposited on the target. In the final step before MALDI-ToF, a tenth of the dissolved sample was mixed with the matrix layer on the target, leaving the rest for other analyses, e.g. chemically assisted fragmentation-MALDI and LC-MS/MS.

Protein identification using MALDI-ToF, chemically assisted fragmentation-MALDI, and LC-MS/MS

Protein identification by peptide-mass fingerprinting (PMF) was performed on Ettan MALDI-ToF Pro. Data acquisition, spectrum processing, and database searches were performed in automatic mode with internal calibration using trypsin.

To further improve the identification rate, chemically-assisted fragmentation-MALDI was used on proteins not successfully identified by PMF. This technique enables peptide sequence data to be acquired simply, quickly, and with good sensitivity by analyzing the CAF-labeled peptides in post-source decay (PSD) mode on Ettan MALDI-ToF Pro.

Finally, the few spots that were still not identified using MALDI MS were subjected to LC-MS/MS analysis. The tandem mass spectrometric analysis was performed on Finnigan™ LTQ™ linear ion trap mass spectrometer (Thermo Electron Corporation) fitted with Biobasic C18 column (100 × 0.1 mm, Thermo Electron Corporation) running at 1 µl/min flow rate. A fast gradient profile enabled a total analysis time of 20 min during which approximately 5500 scans were acquired using the data-dependent "triple play routine". In this mode, peptides detected in a survey scan are selected for high-resolution Zoom Scan followed by fragmentation by MS/MS. The spectra were then processed automatically by SEQUEST™ to get unambiguous identification based on peptide sequence contained in the product ion spectrum. The built-in features of data dependent acquisition™ and dynamic exclusion™ allows automated collection of useful data and enables efficient handling of coeluting peptides, ensuring maximum sensitivity and sequence coverage for identified proteins.

Results and discussion

As expected when preparing samples from an organ like the liver, the protein concentration in the four lots was about 10 mg/ml (treated 1, 10.02 mg/ml; treated 2, 9.67 mg/ml; treated 3, 10.23 mg/ml; pooled untreated control, 10.40 mg/ml), i.e. 1/100 of the prepared sample was protein. This points to a very high recovery since about 1/10 of organs should consist of protein and the 0.5 g samples were diluted 10 times in 5 ml lysis buffer.

After 2-D gel electrophoresis and image analysis, DeCyder software fully matched 1450 spots between the three different images for each gel. Figure 2 shows the matched spots.

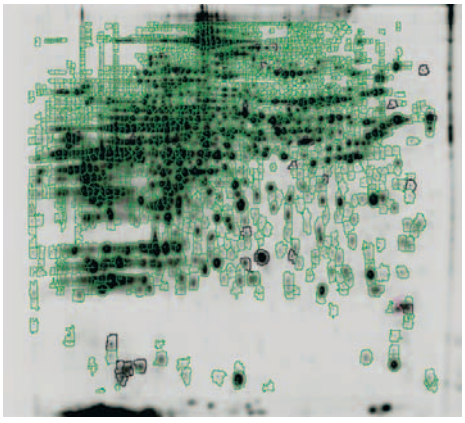

Fig 2. Protein spots (1450) matched on the 2-D gel by DeCyder Differential Analysis Software. A picklist of 552 spots was generated and automatically transferred to Ettan Spot Handling Workstation.

Of these 1450 protein spots, 30 spots were found to be differentially regulated. A total of 552 spots, including the 30 diferentially regulated spots, were selected as a reasonable test for automatic spot handling on the workstation (which can handle a maximum of 1152 samples from up to twelve gels per batch). The picklist was thus generated and automatically transferred to Ettan Spot Handling Workstation, which used it to pick, digest, and spot all 552 proteins, again with full automation and no manual intervention. All 552 spotted proteins gave PMF spectra following preparation in Ettan Spot Handling Workstation. When expectation values <0.05 were collected, 298 of these spots (54%) were automatically identified. Manual editing should increase this number significantly. Figure 3 illustrates the results workflow and shows the methods and outcome of the protein identification.

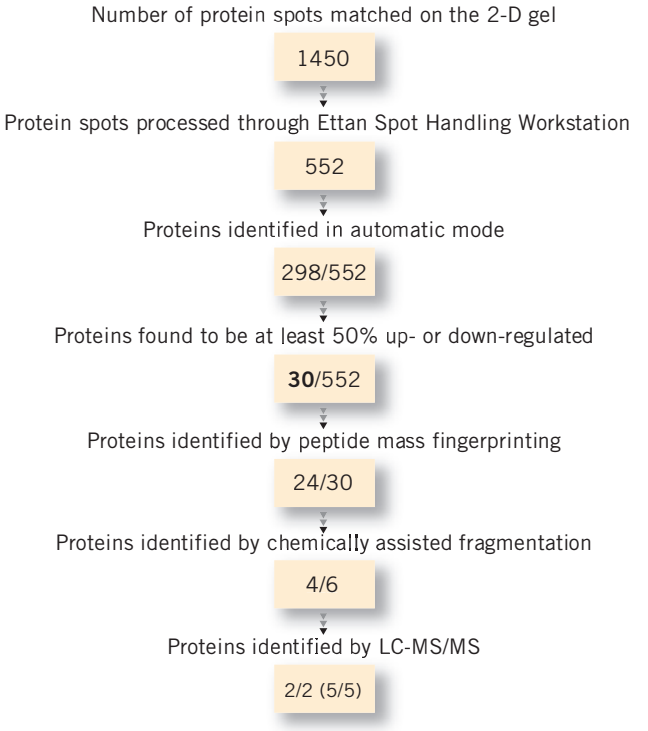
Number of protein spots matched on the 2-D gel
1450
Protein spots processed through Ettan Spot Handling Workstation
552
Proteins identified in automatic mode
298/552
Proteins found to be at least 50% up- or down-regulated
30/552
Proteins identified by peptide mass fingerprinting
24/30
Proteins identified by chemically assisted fragmentation
4/6
Proteins identified by LC-MS/MS
2/2 (5/5)

Fig 3. Workflow from the initial matching of 1450 spots on the 2-D gels to the successful identification of all 30 mouse liver proteins up- or down-regulated by more than 50% following treatment with a candidate drug (LC-MS/MS was used to confirm three identities made by the other two methods).

At this stage, we decided to focus on identifying the regulated 30 proteins. MALDI-ToF analyses identified 24 of the 30 regulated proteins (80%) directly by peptide-mass fingerprinting. Figure 4 shows the spectrum of one of these 24 proteins, later identified as formyl transferase (position 8 in Table 1). Six spots were analyzed by chemically assisted fragmentation-MALDI of which four (67%) were successfully identified (spectra not shown). For the two remaining proteins, an LC-MS/MS method using the Finnigan ProteomeX™ Workstation and LCQ™ Deca XP Plus Ion Trap Mass Spectrometer proved successful (example shown in Fig 5). The LC-MS/MS approach was also used to confirm three identifications made by MALDI-ToF and chemically assisted fragmentation-MALDI. Table 1 shows the identities of all 30 proteins up- or down-regulated by more than 50% following treatment with the candidate drug.

Picking accuracy was high. The backing of the gels stabilizes their dimensions and a self-adhesive label attached prior to scanning links the electronic image with the actual gel. In addition, the design and movement of the picked head ensured a clean and precise cut that also promotes high

picking efficiency. The clean cut, combined with hydrophobic coatings on the picking head and needles, minimized cross-contamination. Carry-over was not detected.

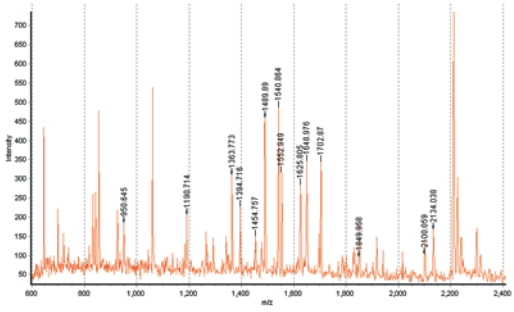

Fig 4. Spectrum of a protein analyzed by MALDI-ToF following automated handling and preparation on Ettan Spot Handling Workstation. The protein was identified as formyl transferase (see Table 1, row 8).

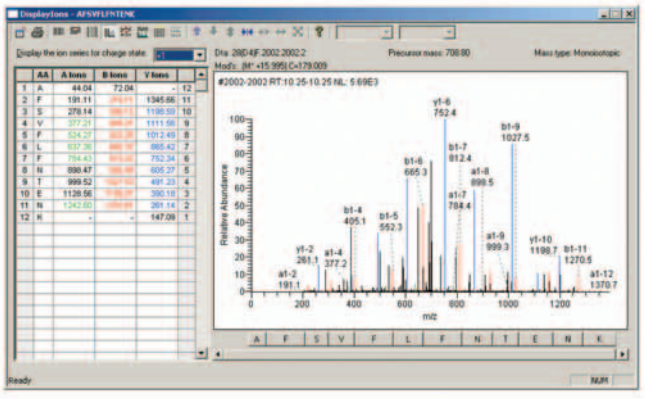

Fig 5. Product ion spectrum of peptide AFSVFLFNTENK (isopentenyldiphosphate delta-isomerase from mouse liver, abbreviated to IPP isomerase in Table 1 on rows 27 and 28) with highlighted b- and y-ion series fragments detected in the spectrum.

Table 1. Protein identification results listing ID method, protein name, ID number (NCBI database), and relevant biochemical information. The protein whose spectrum is shown in Figure 4 is found at position 8 in this list. Average ratio shows extent of regulation, negative values indicate down regulation, positive values indicate up regulation.

	ID method	Average Ratio	Name	Protein ID NCBI nr db	Student's t-test (p =)	pI theo-retical	Mol. weight
1	PMF	-1.59	carbamoyl-phosphate synthetase 1	8393186	3.70×10^{-7}	6.33	164580
2	PMF	2.85	carbamoyl-phosphate synthetase 1	23621369	9.00×10^{-8}	6.03	116816
3	PMF	2.65	carbamoyl-phosphate synthetase 1	8393186	1.30×10^{-8}	6.19	116273
4	PMF	1.55	pyruvate carboxylase	7438124	1.10×10^{-8}	6.34	129777
5	PMF	1.74	pyruvate carboxylase	7438124	1.40×10^{-6}	6.34	129777
6	MS/MS	-1.54	ornithine transcarbamylase	762985	9.50×10^{-8}	7.25	108595
7	PMF	-1.69	similar to elongation factor 2	26328763	1.20×10^{-5}	6.31	95257
8	PMF	1.81	Formyl transferase	25050159	9.40×10^{-6}	5.64	99064
9	PMF	-1.61	Formyl transferase	25050159	2.50×10^{-10}	5.64	99064
10	PMF/MSMS	2.75	ubiquilin 1/serine proteinase inhibitor	20072434/15079234	0.0073	4.73	74813
11	CAF + PMF	-1.84	esterase 31 + carboxylesterase 2	20886287/19527178	6.90×10^{-10}	5.86	72638
12	PMF	-1.68	Liver carboxylesterase precursor	2494382	3.10×10^{-8}	5.88	60406
13	PMF	1.90	T-complex protein 1	22654291	3.40×10^{-6}	5.97	57477
14	PMF	1.56	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	20988709	4.70×10^{-5}	5.65	57569
15	PMF	-2.25	selenium binding protein 2	9507079	3.40×10^{-10}	5.78	52628
16	PMF	1.59	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	12836439	0.0025	8.65	56823
17	PMF	1.66	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	20965433	5.30×10^{-6}	8.65	56823
18	PMF	1.61	methionine adenosyltransferase I	19526790	1.70×10^{-6}	5.51	43509
19	PMF	2.02	methionine adenosyltransferase I	19526790	8.10×10^{-10}	5.51	43509
20	CAF	2.06	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	12836371	2.80×10^{-6}	7.56	56221
21	CAF	1.87	gamma-actin	20885782	0.064*	5.14	50041
22	PMF	1.68	cathepsin D	26354406	3.80×10^{-6}	6.85	48374
23	PMF	1.64	farnesyl diphosphate synthetase	19882207	3.00×10^{-6}	5.48	40582
24	MS/MS	2.59	hepatoma derived growth factor	31560691	0.1*	4.52	40202
25	PMF	-2.22	nudix	12847124	9.80×10^{-9}	5.97	24603
26	PMF	-2.02	thioether S-methyltransferase	6678281	1.60×10^{-10}	6	29460
27	PMF/MS/MS	-1.75	thioether S-methyltransferase/IPP isomerase 1	6678281/13878548	2.40×10^{-8}	5.82	23239
28	CAF/MS/MS	2.21	peroxiredoxin 4/IPP isomerase 1	7948999/13878548	7.00×10^{-9}	6.34	22078
29	PMF	-1.58	Major urinary proteins 11 and 8	127531	5.40×10^{-7}	4.85	17560
30	PMF	-1.54	Major urinary proteins 11 and 8	127531	0.0022	4.85	17560

* Value above p = 0.01. The identity was, however, confirmed by CAF labeling and MS/MS respectively.

Conclusions

We have investigated the suitability of a highly automated proteomics approach to identify differential expression levels of proteins indicated in ADME/Tox processes. Such information should lead to the discovery of useable biomarkers signaling drug toxicity, which would exclude the candidate drug from further development.

This study showed the value of combining products from the Ettan range for sample preparation, differential gel electrophoresis by 2-D DIGE, automatic spot handling, and finally the identification of proteins by a combination of MALDI-ToF PMF, MALDI-ToF PSD of CAF-labeled peptides, and LC-MS/MS.

Ettan Spot Handling Workstation played a central role in the overall investigation. The picklist generated by DeCyder Differential Analysis Software allowed automatic processing of all selected protein spots and made them ready for analysis by MS techniques. All proteins regulated following treatment with the candidate drug were thus successfully detected, selected, prepared, and identified in a smooth, accurate, and highly efficient manner.

The identified proteins were grouped in different functional categories and included proteins that may be involved in the xenobiotic metabolism of drugs, as well as proteins that might be of importance in toxicity.

Further work using narrower and more basic IPG strips to resolve more proteins on 2-D gels is under way. In addition, a focused study on proteins obtained by subcellular fractionation could prove to be very fruitful for further identification of proteins involved in metabolism and toxicity in the liver following administration of drugs. Greater understanding of these processes might significantly improve ADME/Tox investigations during drug discovery campaigns.

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